cue card

Automated multi-step protein purification

ÄKTA 3D Kit

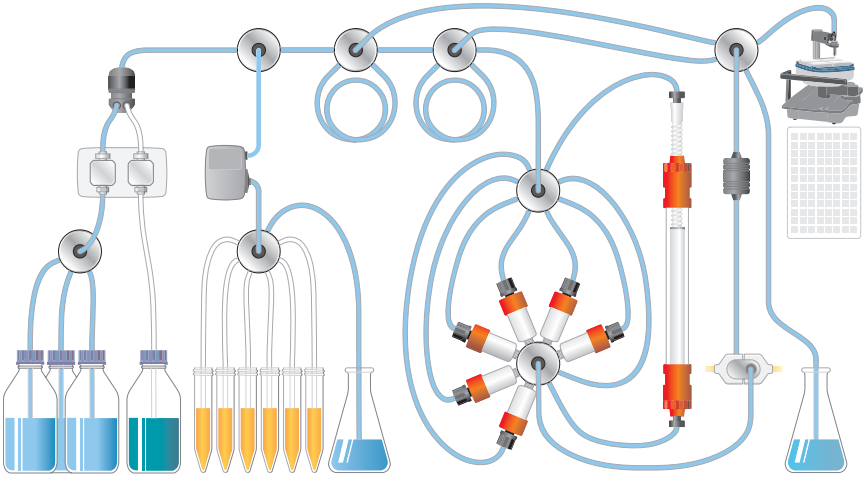
System preparation

Column preparation

Purification protocols

System maintenance

Results and Evaluation



cue 18-1161-47 AB 2002-12 • p1

Amersham Biosciences

System preparations

This cue card describes general system preparations.

To ensure the best results, use high purity water and chemicals. It is also recommended to filter the solvents through a 0.45 µm filter.

System Pump

Fill buffer inlet tubings

- Prepare required buffers according to the chosen purification protocol. Add extra volume for system and column preparation.
- Place the inlet tubings in the buffers according to Figure 1.
- If empty, purge the buffer inlet tubings manually according to Pump P-900 User manual.

Note: When running a purification protocol, system pump wash is included in the method.

Purge system pump

- Open **System Control** and select **Manual:Pump**.
- Mark the instruction **Pump Wash Explorer** and select appropriate pump inlets (e.g. A11, B1, B2). Each pump wash requires approximately 50 ml buffer.
- Press **Execute** to start the wash.
- Press **End** when the wash is completed.

Note: If more than one **InletA1** needs purging (e.g. A11 and A12) the pump wash must be repeated.

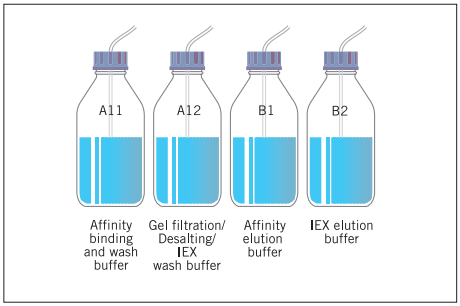


Fig 1. *How to place inlet tubings in buffers when using ÄKTA™ 3D Kit.*

Sample Pump

Fill sample inlet tubings

- Prepare required samples and solutions.
- Place the inlet tubings according to Figure 2.
- Fill sample inlet tubings as follows:

Note: All air bubbles have to be removed from sample inlet tubings.

  – Open **System Control** and select **Manual:FlowPath**.
  – Choose the instruction **SampleValve** and select the appropriate **Position** (e.g. S1) and press **Execute**.
  – Select **Pump** and choose **SampleFlow**.
  – Key in appropriate flow (e.g. 1 ml/min) and press **Execute**.
  – When the selected tubing is filled with solution, press **End**.

- Repeat the procedure above until all tubings are filled and purge the sample pump as described below.

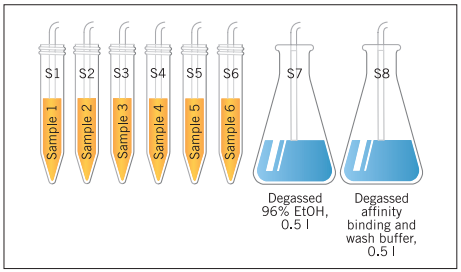


Fig 2. *How to place samples when using ÄKTA 3D Kit.*

Purge sample pump

Purpose: To remove any air introduced in the sample pump while filling the sample inlet tubings. Presence of air bubbles in the pump will affect the flow rate.

Note: Make sure S7 and S8 are filled with degassed EtOH and buffer respectively, as described above.

Create the method

- Open **Method Editor** and select **Use Template** under **File:New**.
- Choose technique **Any**.
- Select the "Purge Sample Pump" template and press **OK**.
- Save the method.

Start the method

The method takes approximately 15 minutes to run. When the method is finished the sample pump flow rate must be verified.

System preparations continued

Verify sample pump flow rate

Purpose: To verify that the sample pump gives correct flow rate. Trapped air in the pump will affect the flow rate. Verifying the flow rate is performed manually from **System Control**.

Note: After each selection below always press **Execute**.

Before starting to measure the flow rate, make sure the outlet tubing F1 is filled with liquid. If not:

- Select **SampleValve S8** in **Manual:Flowpath**.
- Select **ColumnPosition Bypass** in **Manual:Flowpath**.
- Select **InjectionValve Inject** in **Manual:Flowpath**.
- Select **SampleFlow** in **Manual:Pump**, key in e.g. 1ml/min.

When the outlet tubing F1 is filled with liquid press **End**.

Measure flow rate

- Place the outlet tubing F1 in a preweighed beaker.
- Select **Alarm_Pressure** in **Manual:Alarms&Mon** and key in HighAlarm 0.5 MPa.
- Select **SampleValve S8** in **Manual:Flowpath**.
- Select an affinity column e.g. **ColumnPosition 2** in **Manual:Flowpath**.
- Select **DirectLoad** in **Manual:Pump**. Key in a sufficient volume (e.g. 5 ml) and the sample loading flow rate (See Table 1).
- Weigh the beaker containing the collected flowthrough to determine the volume.
- If the volume differs more than 3% from set volume, repeat the "Purge Sample Pump" procedure.
- If the problem remains, see trouble shooting section in the User Manual.

Table 1. *A summary of flow rates (in ml/min) used during sample loading for different columns. RT = room temperature, CR = cold room.*

Column	RT	CR
HiTrap™ Chelating HP, 1 ml	0.5	0.4
HiTrap Chelating HP, 5 ml	5	4
GSTrap™ FF, 1 ml	0.5	0.2
GSTrap FF, 5 ml	2.5	1

Column preparations

This cue card describes how to prepare different columns before running a protocol.

To ensure the best results, use high purity water and chemicals. It is also recommended to filter the solvents through a 0.45 µm filter.

Affinity Columns

Metal ion charging

Purpose: To charge new or stripped HiTrap Chelating HP columns with metal ions (e.g. Ni^{2+}, Co^{2+} or Cu^{2+}). Several columns can be prepared automatically.

Create the method

- Open **Method Editor** and select **Use Template** under **File:New**.
- Choose **Affinity** under **Technique**.
- Select the "Metal Ion Charging" template.
- Select column (HiTrap chelating HP, 1 or 5 ml) and press **OK**.
- Key in appropriate flow rate on the variable page according to Table 1.
- Open the Scouting page. Use **Add** to create the number of runs equal to the number of columns that should be charged.

Note: Even if only one column will be used, a scouting run still has to be added.

- Define column positions for each run.

Note: Default **Column Position** is set to **Position1Bypass**, which means no column.

- Save the method.

Prepare the system

- Prepare required solutions (see Figure 1).
- Connect the chelating columns at defined column positions.
- Place the inlet tubings in the solutions according to Figure 1.
- Fill the inlet tubings S1, S2, S7 and S8 and purge the sample pump according to the instructions in the "System preparations" cue card.

Start the method.

The method takes approximately 15 minutes/column to run.

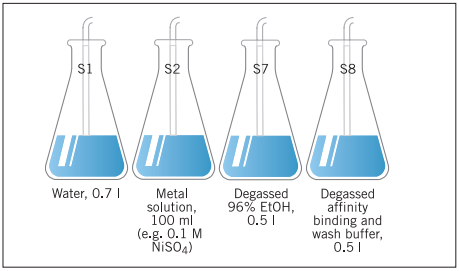


Fig 1. *Solutions required to run the "Metal Ion Charging" template.*

Affinity blank run

Purpose: Prior to a first time usage of an affinity column (GSTrap FF or a newly metal charged HiTrap Chelating HP) it is recommended to run a blank run. This ensures a well-conditioned and equilibrated column ready for chromatography.

Create the method

- Open **Method Editor** and select **Use Template** under **File:New**.
- Choose **Affinity** under **Technique**.
- Select the "Affinity Blank Run" template.
- Select Column and press **OK**.
- Select wash inlets (A11 and B1).
- Key in appropriate flow rates on the variable page according to Table 1.
- Open the Scouting page. Use **Add** to create the number of runs equal to the number of columns that should be equilibrated.

Note: Even if only one column will be used, a scouting run still has to be added.

- Define column positions for each run.

Note: Default **Column Position** is set to **Position1Bypass**, which means no column.

- Save the method.

Table 1. *Recommended flow rates (ml/min) for affinity columns. RT = room temperature, CR = cold room.*

Column	RT	CR
HiTrap Chelating HP, 1 ml	2	1.5
HiTrap Chelating HP, 5 ml	10	8
GSTrap FF, 1 ml	2	1.5
GSTrap FF, 5 ml	10	8

Column preparations continued

Prepare the system

- Prepare required buffers, see Figure 2.
- Place the inlet tubings in the buffers according to Figure 2.
- If empty, purge the inlet tubings (A11 and B1) according to the system pump instructions in the "System preparations" cue card.
- Connect the affinity columns at defined column positions.

Start the method.

The method takes approximately 10 minutes/column.

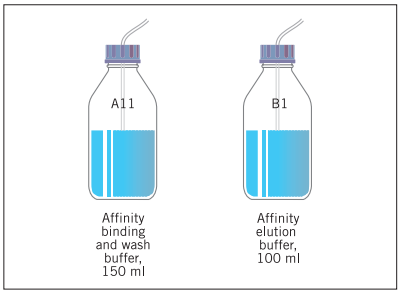


Fig 2. *Buffers required to run the "Affinity Blank Run" template.*

Note: ÄKTA 3D configuration includes a 0.2 MPa flow restrictor. In this method the HighAlarm for the HiTrap columns is set to 0.3 + 0.2 MPa in **Alarm_Pressure** (column pressure limit + flow restrictor). A pressure warning might appear during the affinity blank run, but in this special case the warning can be ignored. The method will continue to run unless the pressure reaches 0.5 MPa.

Ion exchange, Desalting and Gel filtration columns

Equilibrate columns

Purpose: To remove EtOH and equilibrate columns with buffer.

Note: When using a HiLoad™ (gel filtration) or HiPrep™ (desalting) column for the first time or when changing buffer, the column has to be equilibrated.

Prepare the system

- Prepare sufficient volume of the required buffer, see Table 2.
- Place the inlet tubing (e.g. A12) in the equilibration buffer and fill the inlet according to the system pump instructions given in the "System preparation" cue card.
- Connect the columns.

Manual run

Equilibration of columns is performed manually from **System Control**.

Note: After each selection below always press **Execute**.

- Select **Alarm_Pressure** in **Manual:Alarms&Mon**, and key in HighAlarm, see Table 2.
- Select **BufferValveA1** (A12) in **Manual:Flowpath**.
- Select appropriate **ColumnPosition** in **Manual:Flowpath**.
- Select **Flow** in **Manual:Pump**, key in appropriate flow, see Table 2.
- Select **End_timer** in **Manual:Other** and choose **Acc volume**, key in appropriate Timeout volume, see Table 2.
- When the first column is ready, repeat for the next column to be equilibrated.

Note: To make automated methods, see UNICORN™ User Manual.

Table 2. *Recommended HighAlarms, Flow rates and Equilibration volumes for different columns, when running ÄKTA 3D Kit including a 0.2 MPa flow restrictor.*

Column	High Alarm (MPa)	Flow rate removing EtOH (ml/min)	Flow rate using buffer (ml/min)	Timeout Equilibration volume (ml)
HiLoad 16/60	0.5	0.5	1.0	360
HiLoad 26/60	0.5	1.3	2.5	954
HiPrep 26/10	0.35	7.5	15.0	159
HiTrap, 1ml	0.5	0.5	1.0	5
HiTrap, 5ml	0.5	2.5	5.0	25
RESOURCE™, 1ml	1.5	2.0	4.0	5
RESOURCE, 6ml	1.5	3.0	6.0	30
Mono Q™ 5/50 GL	4.0	1.0	2.0	5
Mono S™ 5/50 GL	4.0	1.0	2.0	5

$(His)_6$ Affinity – Gel filtration

This cue card describes how to run this specific purification protocol.

Before starting a purification protocol make sure to prepare system and columns according to the instructions on the system and column preparation cue cards.

1 Prepare your UNICORN method

- Open the method wizard and select purification protocol **C) Affinity (step) – gel filtration**.
- Mark the number of samples **1 to 6** to be purified.
- Indicate your running condition; **room temperature** or **cold room**.
- Press **Next**.
- Choose columns. See Table 1 for recommendations.
- Press **Finish** to obtain the purification method.
- Enter sample volumes on the variable page.
- It is possible to change default values in the method, if needed. Check the "Show details" box. For more details, see User Manual.
- Save your method.

Table 1. *Recommended column combinations.*

Goal	First column		Second column	Frac-950
Up to 10 mg protein	HiTrap Chelating HP, 1 ml	**or**	HiLoad 16/60 Superdex™ 75 prep grade HiLoad 16/60 Superdex 200 prep grade	Rack C with micro titre plates and 50 ml tubes
Up to 50 mg protein	HiTrap Chelating HP, 5 ml	**or**	HiLoad 26/60 Superdex 75 prep grade HiLoad 26/60 Superdex 200 prep grade	Rack A with 10 and 50 ml tubes

2 Prepare your system before a run

- Prepare buffers required for this protein purification protocol. To ensure the best results, use high purity water and chemicals. It is recommended to filter buffers through a 0.45 µm filter.

Examples of buffers for this purification protocol:

Affinity binding and wash buffer: 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 20 mM imidazole

Affinity elution buffer: 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 M imidazole

Gel filtration buffer: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl

- Place buffer inlets as shown in Figure 1 and make sure your system is prepared as described in the "System preparations" cue card.
- Connect selected columns as shown in Figure 2 and prepare them according to the "Column preparations" cue card.
- Depending on which gel filtration column you are running, choose rack C or rack A for the fraction collector, see Table 1. Load the rack with four 96-well microplates (2 ml) or 120 tubes (10 ml) respectively. You will also need to load the fraction collector with 50 ml fraction tubes to collect the second wash of the affinity column (one tube per sample is needed). For more details, see User Manual.

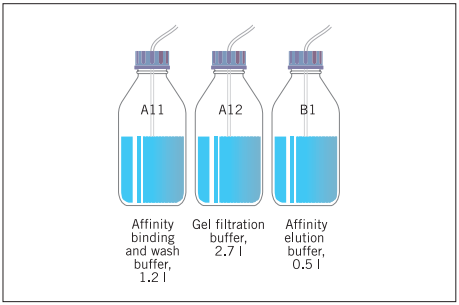


Fig 1. *Buffer inlets and maximum volumes needed, see User Manual for details.*

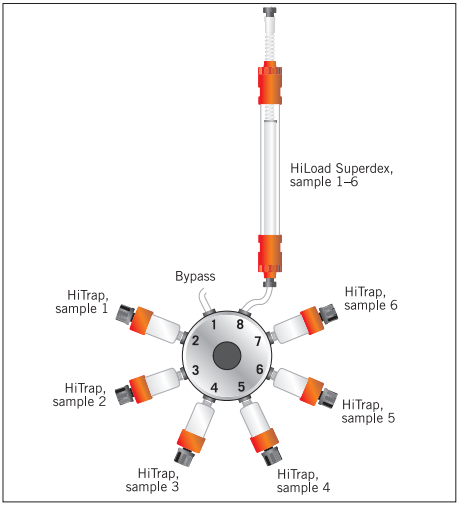


Fig 2. *Column connections at column selection valves V2 and V3.*

$(His)_6$ Affinity – Gel filtration continued

3 Prepare and load samples

- Prepare your samples and clarify them using centrifugation and/or filtration through a 0.45 µm filter.
- Place the samples at the sample inlet valve according to Figure 3 and prepare the sample pump as described in the "System preparations" cue card.

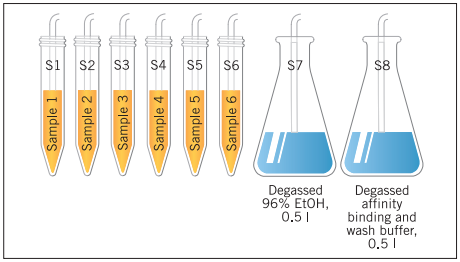


Fig 3. *Sample placement at the sample inlet valve.*

4 Start the method

- Start your prepared method.

In the start protocol:

- Check and change (if necessary) the sample volumes on the variable page.
- Key in sample ID for the proteins to be purified
  - on the variable page (sample ID will be displayed in the chromatogram)
  - on the question page (sample ID can be used in the "**Find**" function to locate results)

Note: You can choose one or both alternatives above for sample ID's.

- Start your automated multi-step purification.

5 Evaluate results

- How to view the results in an easy way, and how to calculate protein concentrations, is described in the Manual and on the "Results and evaluation" cue card.

GST Affinity – Gel filtration

This cue card describes how to run this specific purification protocol.

Before starting a purification protocol make sure to prepare system and columns according to the instructions on the system and column preparation cue cards.

1 Prepare your UNICORN method

- Open the method wizard and select purification protocol **C) Affinity (step) – gel filtration**.
- Mark the number of samples **1 to 6** to be purified.
- Indicate your running condition; **room temperature** or **cold room**.
- Press **Next**.
- Choose columns. See Table 1 for recommendations.
- Press **Finish** to obtain the purification method.
- Enter sample volumes on the variable page.
- It is possible to change default values in the method, if needed. Check the "Show details" box. For more details, see User Manual.
- Save your method.

Table 1. *Recommended column combinations.*

Goal	First column	Second column			Frac-950
Up to 10 mg protein	GSTrap FF, 1 ml	HiLoad 16/60 Superdex 75 prep grade	**or**	HiLoad 16/60 Superdex 200 prep grade	Rack C with micro titre plates and 50 ml tubes
Up to 50 mg protein	GSTrap FF, 5 ml	HiLoad 26/60 Superdex 75 prep grade	**or**	HiLoad 26/60 Superdex 200 prep grade	Rack A with 10 and 50 ml tubes

2 Prepare your system before a run

- Prepare buffers required for this protein purification protocol. To ensure the best results, use high purity water and chemicals. It is recommended to filter buffers through a 0.45 µm filter.

Examples of buffers for this purification protocol:

Affinity binding and wash buffer: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM DTT

Affinity elution buffer: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM DTT, 10 mM reduced glutathione

Gel filtration buffer: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl

- Place buffer inlets as shown in Figure 1 and make sure your system is prepared as described in the "System preparations" cue card.
- Connect selected columns as shown in Figure 2 and prepare them according to the "Column preparations" cue card.
- Depending on which gel filtration column you are running, choose rack C or rack A for the fraction collector, see Table 1. Load the rack with four 96-well microplates (2 ml) or 120 tubes (10 ml) respectively. You will also need to load the fraction collector with 50 ml fraction tubes to collect the second wash of the affinity column (one tube per sample is needed). For more details, see User Manual.

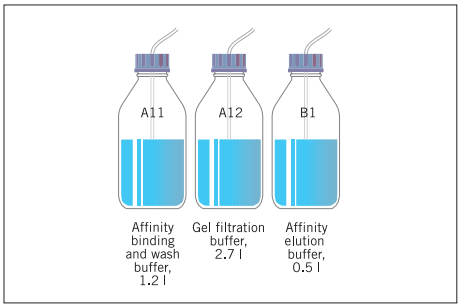


Fig 1. *Buffer inlets and maximum volumes needed, see User Manual for details.*

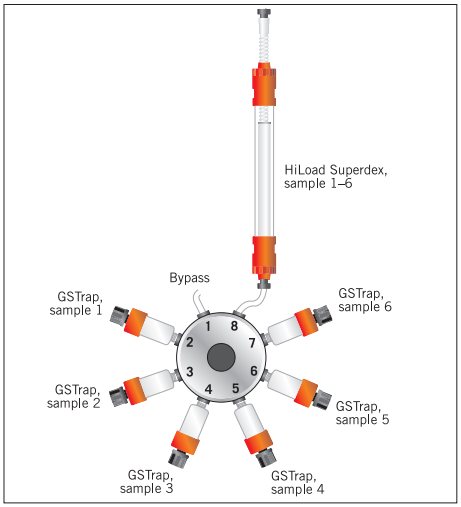


Fig 2. *Column connections at column selection valves V2 and V3.*

GST Affinity – Gel filtration continued

3 Prepare and load samples

- Prepare your samples and clarify them using centrifugation and/or filtration through a 0.45 µm filter.
- Place the samples at the sample inlet valve according to Figure 3 and prepare the sample pump as described in the "System preparations" cue card.

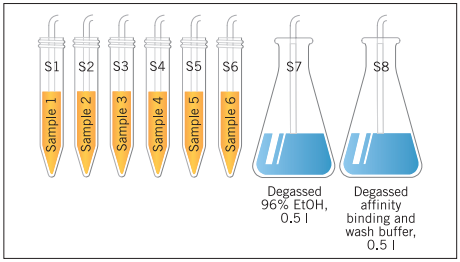


Fig 3. *Sample placement at the sample inlet valve.*

4 Start the method

- Start your prepared method.

In the start protocol:

- Check and change (if necessary) the sample volumes on the variable page.
- Key in sample ID for the proteins to be purified
  - on the variable page
    (sample ID will be displayed in the chromatogram)
  - on the question page
    (sample ID can be used in the "**Find**" function to locate results)

Note: You can choose one or both alternatives above for sample ID's.

- Start your automated multi-step purification.

5 Evaluate results

- How to view the results in an easy way, and how to calculate protein concentrations, is described in the Manual and on the "Results and evaluation" cue card.

$(His)_6$ Affinity – Desalting – AIEX (anion IEX)

For proteins with low pI

This cue card describes how to run this specific purification protocol.

Before starting a purification protocol make sure to prepare system and columns according to the instructions on the system and column preparation cue cards.

1 Prepare your UNICORN method

- Open the method wizard and select purification protocol **E) Affinity (step) – desalting – IEX**.
- Mark the number of samples **1 to 4** to be purified.
- Indicate your running condition; **room temperature** or **cold room**.
- Press **Next**.
- Choose columns. See Table 1 for recommendations.
- Press **Finish** to obtain the purification method.
- Enter sample volumes on the variable page.
- It is possible to change default values in the method, if needed. Check the "Show details" box. For more details, see User Manual.
- Save your method.

Table 1. *Recommended column combinations.*

Goal	First column	Second column	Third column	Frac-950
Up to 10 mg protein	HiTrap Chelating HP, 1 ml	2x5 ml, HiTrap Desalting or HiPrep 26/10 Desalting	1, 5 or 6 ml AIEX	Rack C with micro titre plates and 50 ml tubes
Up to 50 mg protein	HiTrap Chelating HP, 5 ml	HiPrep 26/10 Desalting	5 or 6 ml AIEX	Rack C with micro titre plates and 50 ml tubes

2 Prepare your system before a run

- Prepare buffers required for this protein purification protocol. To ensure the best results, use high purity water and chemicals. It is recommended to filter buffers through a 0.45 µm filter.

Examples of buffers for this purification protocol:

Affinity binding and wash buffer: 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 20 mM imidazole

Affinity elution buffer: 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 M imidazole

Desalting and AIEX binding buffer: 50 mM Tris-HCl pH 8.0

AIEX elution buffer: 50 mM Tris-HCl pH 8.0, 1 M NaCl

- Place buffer inlets as shown in Figure 1 and make sure your system is prepared as described in the "System preparations" cue card.
- Connect selected columns as in Figure 2 and prepare them according to the "Column preparations" cue card.
- Load the rack with four 96-well microplates (2 ml). You will also need to load the fraction collector with 50 ml fraction tubes to collect the second wash of the affinity column and the IEX flowthrough. (Two tubes per sample are needed). For more details, see User Manual.

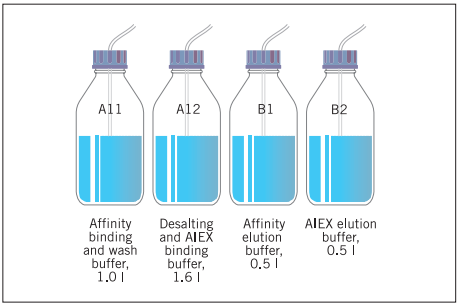


Fig 1. *Buffer inlets and maximum volumes needed, see User Manual for details.*

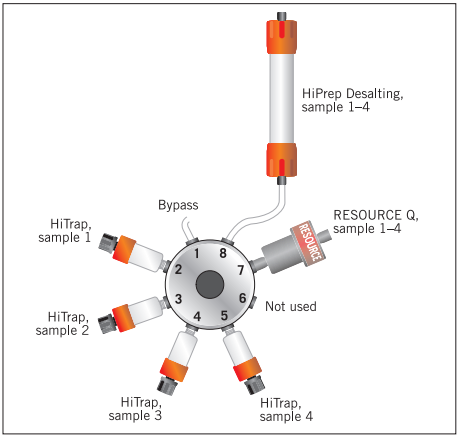


Fig 2. *Column connections at column selection valves V2 and V3.*

$(His)_6$ Affinity – Desalting – AIEX continued

3 Prepare and load samples

- Prepare your samples and clarify them using centrifugation and/or filtration through a 0.45 µm filter.
- Place the samples at the sample inlet valve according to Figure 3 and prepare the sample pump as described in the "System preparations" cue card.

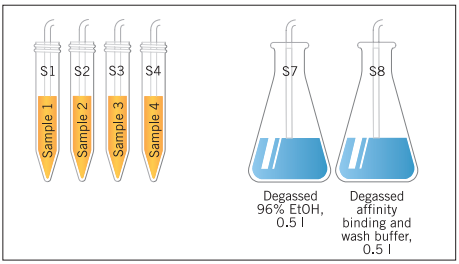


Fig 3. *Sample placement at the sample inlet valve.*

4 Start the method

- Start your prepared method.

In the start protocol:

- Check and change (if necessary) the sample volumes on the variable page.
- Key in sample ID for the proteins to be purified
  - on the variable page (sample ID will be displayed in the chromatogram)
  - on the question page (sample ID can be used in the "**Find**" function to locate results)

Note: You can choose one or both alternatives above for sample ID's.

- Start your automated multi-step purification.

5 Evaluate results

- How to view the results in an easy way, and how to calculate protein concentrations, is described in the Manual and on the "Results and evaluation" cue card.

$(His)_6$ Affinity – Desalting – CIEX (cation IEX)

For proteins with high pI

This cue card describes how to run this specific purification protocol.

Before starting a purification protocol make sure to prepare system and columns according to the instructions on the system and column preparation cue cards.

1 Prepare your UNICORN method

- Open the method wizard and select purification protocol **E) Affinity (step) – desalting – IEX**.
- Mark the number of samples **1 to 4** to be purified.
- Indicate your running condition; **room temperature** or **cold room**.
- Press **Next**.
- Choose columns. See Table 1 for recommendations.
- Press **Finish** to obtain the purification method.
- Enter sample volumes on the variable page.
- It is possible to change default values in the method, if needed. Check the "Show details" box. For more details, see User Manual.
- Save your method.

Table 1. *Recommended column combinations.*

Goal	First column	Second column	Third column	Frac-950
Up to 10 mg protein	HiTrap Chelating HP, 1 ml	2x5 ml, HiTrap Desalting or HiPrep 26/10 Desalting	1, 5 or 6 ml CIEX	Rack C with micro titre plates and 50 ml tubes
Up to 50 mg protein	HiTrap Chelating HP, 5 ml	HiPrep 26/10 Desalting	5 or 6 ml CIEX	Rack C with micro titre plates and 50 ml tubes

2 Prepare your system before a run

- Prepare buffers required for this protein purification protocol. To ensure the best results, use high purity water and chemicals. It is recommended to filter buffers through a 0.45 µm filter.

Examples of buffers for this purification protocol:

Affinity binding and wash buffer: 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 20 mM imidazole

Affinity elution buffer: 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.5 M imidazole

Desalting and CIEX binding buffer: 20 mM Mes pH 6.0

CIEX elution buffer: 20 mM Mes pH 6.0, 1 M NaCl

- Place buffer inlets as shown in Figure 1 and make sure your system is prepared as described in the "System preparations" cue card.
- Connect selected columns as in Figure 2 and prepare them according to the "Column preparations" cue card.
- Load the rack with four 96-well microplates (2 ml). You will also need to load the fraction collector with 50 ml fraction tubes to collect the second wash of the affinity column and the IEX flowthrough. (Two tubes per sample are needed). For more details, see User Manual.

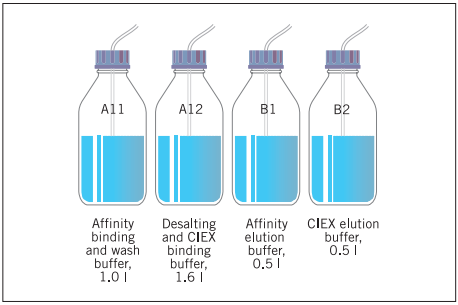


Fig 1. *Buffer inlets and maximum volumes needed, see User Manual for details.*

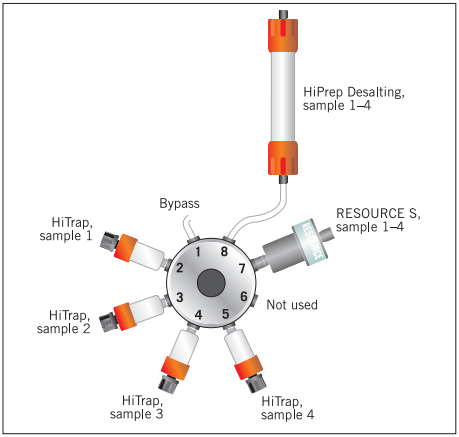


Fig 2. *Column connections at column selection valves V2 and V3.*

$(His)_6$ Affinity – Desalting – CIEX continued

3 Prepare and load samples

- Prepare your samples and clarify them using centrifugation and/or filtration through a 0.45 µm filter.
- Place the samples at the sample inlet valve according to Figure 3 and prepare the sample pump as described in the "System preparations" cue card.

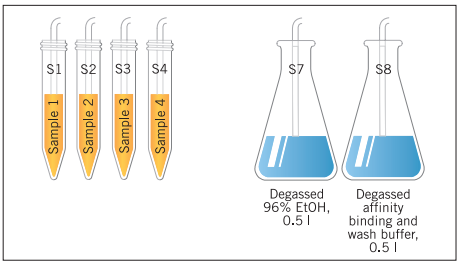


Fig 3. *Sample placement at the sample inlet valve.*

4 Start the method

- Start your prepared method.

In the start protocol:

- Check and change (if necessary) the sample volumes on the variable page.
- Key in sample ID for the proteins to be purified
  - on the variable page
    (sample ID will be displayed in the chromatogram)
  - on the question page
    (sample ID can be used in the "**Find**" function to locate results)

Note: You can choose one or both alternatives above for sample ID's.

- Start your automated multi-step purification.

5 Evaluate results

- How to view the results in an easy way, and how to calculate protein concentrations, is described in the Manual and on the "Results and evaluation" cue card.

GST Affinity – Desalting – AIEX (anion IEX)

For proteins with low pI

This cue card describes how to run this specific purification protocol.

Before starting a purification protocol make sure to prepare system and columns according to the instructions on the system and column preparation cue cards.

1 Prepare your UNICORN method

- Open the method wizard and select purification protocol **E) Affinity (step) – desalting – IEX**.
- Mark the number of samples **1 to 4** to be purified.
- Indicate your running condition; **room temperature** or **cold room**.
- Press **Next**.
- Choose columns. See Table 1 for recommendations.
- Press **Finish** to obtain the purification method.
- Enter sample volumes on the variable page.
- It is possible to change default values in the method, if needed. Check the "Show details" box. For more details, see User Manual.
- Save your method.

Table 1. *Recommended column combinations.*

Goal	First column	Second column	Third column	Frac-950
Up to 10 mg protein	GSTrap FF, 1 ml	2x5 ml, HiTrap Desalting or HiPrep 26/10 Desalting	1, 5 or 6 ml AIEX	Rack C with micro titre plates and 50 ml tubes
Up to 50 mg protein	GSTrap FF, 5 ml	HiPrep 26/10 Desalting	5 or 6 ml AIEX	Rack C with micro titre plates and 50 ml tubes

2 Prepare your system before a run

- Prepare buffers required for this protein purification protocol. To ensure the best results, use high purity water and chemicals. It is recommended to filter buffers through a 0.45 µm filter.

Examples of buffers for this purification protocol:

Affinity binding and wash buffer: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM DTT

Affinity elution buffer: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM DTT, 10 mM reduced glutathione

Desalting and AIEX binding buffer: 50 mM Tris-HCl pH 8.0

AIEX elution buffer: 50 mM Tris-HCl pH 8.0, 1 M NaCl

- Place buffer inlets as shown in Figure 1 and make sure your system is prepared as described in the "System preparations" cue card.
- Connect selected columns as in Figure 2 and prepare them according to the "Column preparations" cue card.
- Load the rack with four 96-well microplates (2 ml). You will also need to load the fraction collector with 50 ml fraction tubes to collect the second wash of the affinity column and the IEX flowthrough. (Two tubes per sample are needed). For more details, see User Manual.

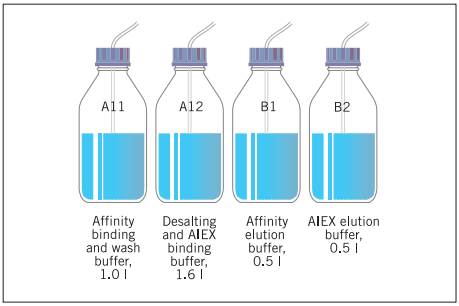


Fig 1. *Buffer inlets and maximum volumes needed, see User Manual for details.*

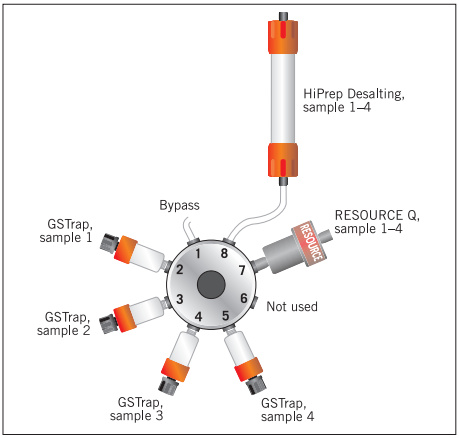


Fig 2. *Column connections at column selection valves V2 and V3.*

GST Affinity – Desalting – AIEX continued

3 Prepare and load samples

- Prepare your samples and clarify them using centrifugation and/or filtration through a 0.45 µm filter.
- Place the samples at the sample inlet valve according to Figure 3 and prepare the sample pump as described in the "System preparations" cue card.

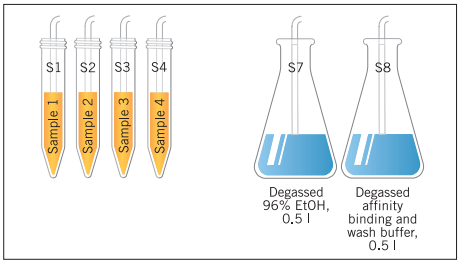


Fig 3. *Sample placement at the sample inlet valve.*

4 Start the method

- Start your prepared method.

In the start protocol:

- Check and change (if necessary) the sample volumes on the variable page.
- Key in sample ID for the proteins to be purified
  - on the variable page (sample ID will be displayed in the chromatogram)
  - on the question page (sample ID can be used in the "**Find**" function to locate results)

Note: You can choose one or both alternatives above for sample ID's.

- Start your automated multi-step purification.

5 Evaluate results

- How to view the results in an easy way, and how to calculate protein concentrations, is described in the Manual and on the "Results and evaluation" cue card.

GST Affinity – Desalting – CIEX (cation IEX)

For proteins with high pI

This cue card describes how to run this specific purification protocol.

Before starting a purification protocol make sure to prepare system and columns according to the instructions on the system and column preparation cue cards.

1 Prepare your UNICORN method

- Open the method wizard and select purification protocol **E) Affinity (step) – desalting – IEX**.
- Mark the number of samples **1 to 4** to be purified.
- Indicate your running condition; **room temperature** or **cold room**.
- Press **Next**.
- Choose columns. See Table 1 for recommendations.
- Press **Finish** to obtain the purification method.
- Enter sample volumes on the variable page.
- It is possible to change default values in the method, if needed. Check the "Show details" box. For more details, see User Manual.
- Save your method.

Table 1. *Recommended column combinations.*

Goal	First column	Second column	Third column	Frac-950
Up to 10 mg protein	GSTrap FF, 1 ml	2x5 ml, HiTrap Desalting or HiPrep 26/10 Desalting	1, 5 or 6 ml CIEX	Rack C with micro titre plates and 50 ml tubes
Up to 50 mg protein	GSTrap FF, 5 ml	HiPrep 26/10 Desalting	5 or 6 ml CIEX	Rack C with micro titre plates and 50 ml tubes

2 Prepare your system before a run

- Prepare buffers required for this protein purification protocol. To ensure the best results, use high purity water and chemicals. It is recommended to filter buffers through a 0.45 µm filter.

Examples of buffer choices for this purification protocol:

Affinity binding and wash buffer: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM DTT

Affinity elution buffer: 50 mM Tris-HCl pH 8, 0.15 M NaCl, 1 mM DTT, 10 mM reduced glutathione

Desalting and CIEX binding buffer: 20 mM Mes pH 6.0

CIEX elution buffer: 20 mM Mes pH 6.0, 1 M NaCl

- Place buffer inlets as shown in Figure 1 and make sure your system is prepared as described in the "System preparations" cue card.
- Connect selected columns as in Figure 2 and prepare them according to the "Column preparations" cue card.
- Load the rack with four 96-well microplates (2 ml). You will also need to load the fraction collector with 50 ml fraction tubes to collect the second wash of the affinity column and the IEX flowthrough. (Two tubes per sample are needed). For more details, see User Manual.

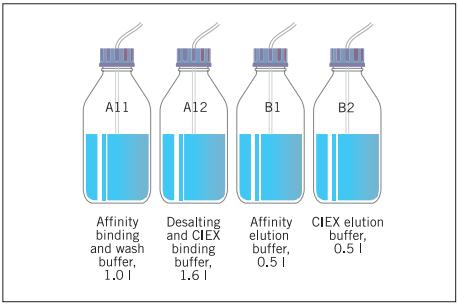


Fig 1. *Buffer inlets and maximum volumes needed, see User Manual for details.*

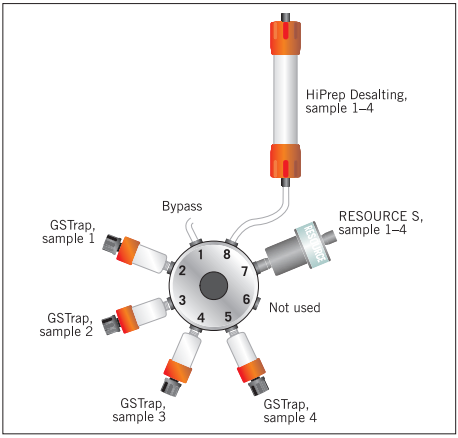


Fig 2. *Column connections at column selection valves V2 and V3.*

GST Affinity – Desalting – CIEX continued

3 Prepare and load samples

- Prepare your samples and clarify them using centrifugation and/or filtration through a 0.45 µm filter.
- Place the samples at the sample inlet valve according to Figure 3 and prepare the sample pump as described in the "System preparations" cue card.

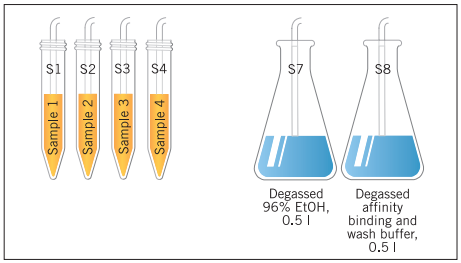


Fig 3. *Sample placement at the sample inlet valve.*

4 Start the method

- Start your prepared method.

In the start protocol:

- Check and change (if necessary) the sample volumes on the variable page.
- Key in sample ID for the proteins to be purified
  - on the variable page (sample ID will be displayed in the chromatogram)
  - on the question page (sample ID can be used in the "**Find**" function to locate results)

Note: You can choose one or both alternatives above for sample ID's.

- Start your automated multi-step purification.

5 Evaluate results

- How to view the results in an easy way, and how to calculate protein concentrations, is described in the Manual and on the "Results and evaluation" cue card.

System maintenance and column cleaning

This cue card describes how to clean your system and columns after performed protein purification.

To ensure the best results, use high purity water and chemicals. It is also recommended to filter buffers through a 0.45 µm filter.

System and Loop Wash

Purpose: To wash all used tubings within the system, including the loops.

Create the method

- Open Method Editor and select **Use Template** under **File:New**.
- Select the "System and Loop Wash" template and press **OK**.
- Select up to two buffer inlets to be washed at the same time as the system (e.g. A11 and B1).

Note: If more than one **InletA1** needs washing (e.g. A11 and A12) we recommend to clean these inlets according to the "System Pump" instructions in the "System preparations" cue card before performing the System Wash.

Sample inlet tubings must be washed separately.

- Save the method.

Prepare the system

- Prepare a sufficient volume (0.4 l) of the wash solution.
- Place the inlet tubings in the wash solution.

Start the method.

The method takes approximately 10 minutes to run.

Column CIP (cleaning-in-place)

Purpose: To clean contaminated columns. For column cleaning procedures and column storage instructions, please refer to the instructions supplied with each column or to our homepage: *www.chromatography.amershambiosciences.com*

Note: HiTrap columns are disposable. If they become dirty or clogged they should be replaced.

Create the method

- Open Method Editor and select **Use Template** under **File:New**.
- Select the "Column CIP" template.
- Select **Column** and press **OK**.

Note: If you wish to CIP different types of columns using the same solutions and method you can change this later by changing column on the Scouting page.

- Open the Scouting page. Use **Add** to create the number of runs equal to the number of columns that should be cleaned.

Note: Even if only one column will be used, a scouting run still has to be added.

- Define **Column** and **Column Position** for each run.
- Select the buffer inlets, flow rates and CIP volumes for all solutions that will be used. Up to 10 steps per column can be performed.
- Save the method.

Prepare the system

- Prepare a sufficient volume of each required solution.
- Connect the columns at defined column positions.
- Place the inlet tubings in appropriate solutions.
- Fill the inlet tubings according to "System Pump" instructions in the "System preparations" cue card.

Start the method.

System maintenance and column cleaning continued

Metal Ion Stripping (chelating columns)

Purpose: To remove metal ions before regenerating the HiTrap HP columns. To recharge the column, follow the instructions given on the "Column preparations" cue card.

Note: Always remove metal ions before or right after storing the HiTrap Chelating columns in EtOH.

Create the method

- Open Method Editor and select **Use Template** under **File:New**.
- Choose technique **Affinity**.
- Select the "Metal Ion Stripping" template.
- Select **Column** and press **OK**.
- Choose flow rates on the variable page according to Table 1.
- Open the Scouting page. Use **Add** to create the number of runs equal to the number of columns that should be stripped.

Note: Even if only one column will be used, a scouting run still has to be added.

- Define **Column Position** for each run.
- Save the method.

Table 1. *Recommended flow rates (ml/min) for stripping the HiTrap Chelating columns. RT = room temperature, CR = cold room.*

Column	RT	CR
HiTrap Chelating HP, 1 ml	2	1.5
HiTrap Chelating HP, 5 ml	10	8

Prepare the system

- Prepare required solutions, see Figure 1.
- Connect the columns at defined column positions.
- Place the inlet tubings in the solutions according to Figure 1.
- Fill the inlet tubings S1, S3, S7 and S8, and purge the sample pump according to instructions in the "System preparations" cue card.

Note: If the "Metal Ion Charging" template will be run afterwards, it is useful to also fill the inlet tubing S2 with metal solution at this time (see "Column preparations" cue card).

Start the method.

The method takes approximately 20 minutes/column to run.

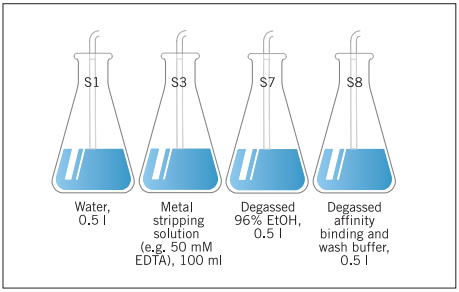
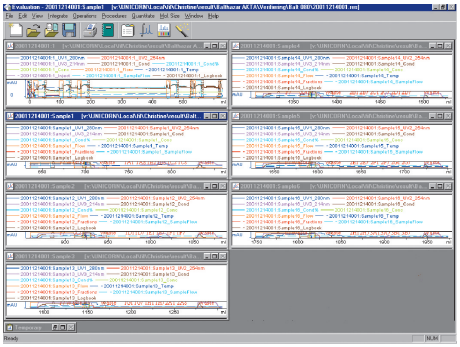


Fig 1. *Solutions required for the "Metal Ion Stripping" template.*

Results and evaluation

Finding and navigating results



- Open the result file directly or search the result files for sample ID's by using the **File:Find** function in UNICORN Main Menu.
- In the evaluation module right-click on the chromatogram for Sample1 and select **Properties**.
- In the curve selection table select curves to be displayed (commonly; **1**, **4**, **6**, **11** and **22**). (If the **Clear** function is used, remember to also scroll down and select curve **22**.)
- Use the function **Apply to all chromatograms** to automatically transfer these settings to all other chromatograms.
- Press **OK** to confirm your selections.

The first chromatogram shows the loading of all samples onto the affinity columns. Each one of the remaining chromatograms shows the purification of one individual sample. When only one sample is purified the result file will only contain one chromatogram that includes both loading and purification.

The sample order corresponds to the sample ID entered in **Questions** before the run was started.

Note: If you have entered the sample ID on the variable page, the sample ID will be shown in the chromatogram heading.

When a sample is located press to maximize the chromatogram.

When using the normal print function, all chromatograms will be printed. Read the User Manual about how to print a single chromatogram.

Determination of protein concentration and amount

For calculating sample concentrations and amounts the functions **Pool Fractions** and **Fraction histogram** are useful tools.

- Maximize the sample window and zoom in on the relevant fractions.
- Use the function **Pool Fractions** in the Operations menu and select the fractions that are to be pooled.

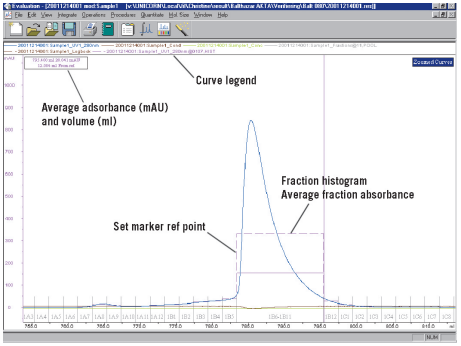
A new fraction mark curve is created and displayed.

- Deselect (right mouse click; select properties) the original fraction mark curve to ease inspection.
- Select **Fraction Histogram** in the Operations menu.
- Select the fraction pool in the **Fraction marks** field.

Depending on what UNICORN version you are using, follow the calculation procedures below.

If you are using UNICORN version 4.0

The fraction histogram function creates a curve that shows the average UV_{280}-absorbance for each fraction or pool.



- Right-click on the chromatogram and select **Marker**.
- To switch to the histogram curve click on the curve legend in the upper part of the chromatogram.
- Move the marker to the area where the fraction pool is located. Note the average UV- absorbance value in the upper left corner of the chromatogram.

Results and evaluation continued

- Place the marker on the left side of the pooled fractions, right-click and select **Set Marker Ref. Point**.
- Move the marker to the right side of the pooled fractions. Note the pooled fraction volume (**x** ml From ref.) shown in the upper left corner of the chromatogram.
- Use the formulas below to calculate protein concentration and total protein amount.

Protein concentration

Conc = Abs / (d *1000* E_{coeff})

Depending on the unit of the used protein extinction coefficient the protein concentration will be expressed in M (mol/l) or mg/ml.

Protein extinction coefficient at 280nm

$E_{coeff} = \varepsilon_M$ (unit: M_{-1} cm_{-1} or l mol_{-1} cm_{-1})

or

$E_{coeff} = E_{0.1\%/1\ cm}$ (corresponds to 1 g/l (1mg/ml))

Abs = average fraction absorbance (mAU)

d = UV-cell path length in cm

(See Appendix B, Measuring the UV-900 flow cell length, in the User Manual for exact determination of the UV cell path length).

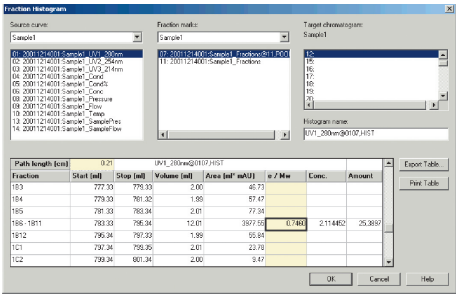
Note: If the real cell length has been set directly in Monitor UV-900 (possible in module software version 2.0 and higher) enter the nominal cell length (d=0.2 cm) above. For more information see User Manual.

Total protein amount

Amount (mg) = $Conc_{mg/ml}$ * fraction pool volume (ml)

If you are using UNICORN version 4.11

UNICORN version 4.11 includes functions for determining the protein concentration and amount automatically.



- Enter real cell length of the UV cell in the **Path length [cm]** field

(See Appendix B, Measuring the UV-900 flow cell length, in the User Manual for exact determination of the UV cell path length).

Note: If the real cell length has been set directly in Monitor UV-900 (possible in module software version 2.0 and higher) enter the nominal path length (0.2 cm) above. For more information see User Manual.

- Enter the protein extinction coefficient at 280 nm in the **e / Mw** column for the selected fraction or pool.

Depending on the unit of the used protein extinction coefficient the protein concentration will be expressed in M (mol/l) or mg/ml.

Protein extinction coefficient at 280 nm

$E_{koeff} = \varepsilon_M$ (unit: M_{-1} cm_{-1} or l mol_{-1} cm_{-1})

or

$E_{koeff} = E_{0.1\%/1\ cm}$ (corresponds to 1 g/l (1mg/ml))

The protein concentration of the fraction or pool is shown in the **Conc.** column.

The total protein amount of the fraction or pool is shown in the **Amount** column.

To convert between Molar and mg/ml use the proteins molecular weight (Da, i.e. g/mol) as shown ⇒ $Conc_{mg/ml} = Conc_M$ * Molecular weight (Da)
$Conc_M = Conc_{mg/ml}$ / Molecular weight (Da)

Ordering information

Product	Pack Size	Code Number
ÄKTA 3D Kit*, with software, valve (INV-907), tubings, sample loops, fittings, instruction manual and cue cards	1	18-1170-00
ÄKTAexplorer 100	please contact your local sales representative of Amersham Biosciences	
Fraction collector Frac-950, complete with Rack A, 18 and 30 mm tubes	1	18-6083-00
Rack C, complete with bowl for 96-well microtitre plates** and 30 mm tubes	1	18-6083-13
Supported Columns:		
Affinity chromatography		
HiTrap Chelating HP	5 x 1 ml	17-0408-01
HiTrap Chelating HP	1 x 5 ml	17-0409-01
GSTrap FF	5 x 1 ml	17-5130-01
GSTrap FF	2 x 1 ml	17-5130-02
GSTrap FF	1 x 5 ml	17-5131-01
Gel filtration		
HiLoad 16/60 Superdex 75 prep grade	1 x 120 ml	17-1068-01
HiLoad 16/60 Superdex 200 prep grade	1 x 120 ml	17-1069-01
HiLoad 26/60 Superdex 75 prep grade	1 x 318 ml	17-1070-01
HiLoad 26/60 Superdex 200 prep grade	1 x 318 ml	17-1071-01
Buffer exchange		
HiPrep 26/10 Desalting	1 x 53 ml	17-5087-01
HiTrap Desalting	5 x 5 ml	17-1408-01
Ion exchange chromatography		
RESOURCE Q	1 x 1 ml	17-1177-01
RESOURCE Q	1 x 6 ml	17-1179-01
RESOURCE S	1 x 1 ml	17-1178-01
RESOURCE S	1 x 6 ml	17-1180-01
HiTrap Q HP	5 x 1 ml	17-1153-01
HiTrap Q HP	5 x 5 ml	17-1154-01
HiTrap SP HP	5 x 1 ml	17-1151-01
HiTrap SP HP	5 x 5 ml	17-1152-01
HiTrap Q FF	5 x 1 ml	17-5053-01
HiTrap Q FF	5 x 5 ml	17-5156-01
HiTrap SP FF	5 x 1 ml	17-5054-01
HiTrap SP FF	5 x 5 ml	17-5157-01
HiTrap DEAE FF	5 x 1 ml	17-5055-01
HiTrap DEAE FF	5 x 5 ml	17-5154-01
HiTrap CM FF	5 x 1 ml	17-5056-01
HiTrap CM FF	5 x 5 ml	17-5155-01
HiTrap ANX FF (high sub)	5 x 1 ml	17-5162-01
HiTrap ANX FF (high sub)	5 x 5 ml	17-5163-01
HiTrap Q XL	5 x 1 ml	17-5158-01
HiTrap Q XL	5 x 5 ml	17-5159-01
HiTrap SP XL	5 x 1 ml	17-5160-01
HiTrap SP XL	5 x 5 ml	17-5161-01
Mono Q 5/50 GL	1 x 1 ml	17-5166-01
Mono S 5/50 GL	1 x 1 ml	17-5168-01
Related products and literature:		
ÄKTA 3D Kit User Manual	1	18-1161-46
Test Kit 280 nm (2 mm cell)	1	18-1129-63
ÄKTA 3D Kit Miniposter	1	18-1160-46
HiTrap Column Guide	1	18-1129-81
ÄKTAdesign Brochure	1	18-1158-77
ÄKTA 3D Kit Brochure	1	18-1160-45
ÄKTAexplorer chromatography systems Data File	1	18-1124-09
Fraction collector Frac-950 Data File	1	18-1153-57
Affinity Chromatography Handbook	1	18-1022-29
Gel Filtration Handbook	1	18-1022-18
Ion Exchange Chromatography Handbook	1	18-1114-21
Recombinant Protein Handbook	1	18-1142-75
GST Gene Fusion System Handbook	1	18-1157-58

* ÄKTAexplorer100 (with Pump P-950 and UNICORN 4.0 or higher) and Fraction collector Frac-950 (with Rack A and Rack C; Rack A is the standard rack supplied with Fraction collector Frac-950) are needed to use ÄKTA 3D Kit. ÄKTAexplorer 100 upgrades can be ordered from your local Labcrew™ representative of Amersham Biosciences. A representative of Amersham Biosciences is required to install ÄKTA 3D Kit.

** Amersham Biosciences recommends Greiner, PP-Masterblock, 2 ml, 96 well: 780270

Trouble shooting and useful hints can be found in the User Manual: 18-1161-46 AB.

to order:

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